



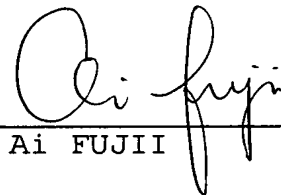
## DECLARATION

I, Ai FUJII, of HIRAKI & ASSOCIATES, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and Japanese languages and am competent to translate from Japanese into English.

2. That I have executed, with the best of my ability, a true and correct translation into English of Japanese Patent Application No. 2003-21047 filed on January 29, 2003, a copy of which I attach herewith.

This 12th day of June, 2007

  
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Ai FUJII

English Translation of Previous Application

Country: Japan

Type: Patent

Date of Application: January 29, 2003

Application Number: No. 2003-21047

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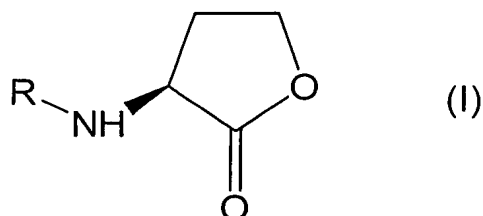
[Name of Document] DESCRIPTION

[Title of the Invention] APOPTOSIS INDUCER

[Scope of the Claim]

[Claim 1] An Akt inhibitor comprising a compound represented by formula I:

[Formula 1]



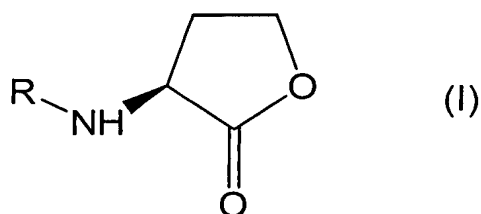
[wherein R is C<sub>4-30</sub> linear or branched acyl, which may be substituted].

[Claim 2] The Akt inhibitor of claim 1, wherein R is C<sub>4-30</sub> linear or branched acyl having oxo at position 3.

[Claim 3] An agent for inducing apoptosis, comprising an Akt inhibitor of claim 1 or 2.

[Claim 4] A method of inducing apoptosis in cells, comprising using a compound represented by formula I:

[Formula 2]



[wherein R is as defined in claim 1].

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a compound inhibiting Akt, which is a type of kinase, an agent for inducing apoptosis comprising the compound.

[0002]

[Prior Art]

Apoptosis is the process of physiological cell death that has been proposed by Kerr and Wyllie et al., (see Non Patent Documents 1 and 2). Apoptosis is not the simple phenomenon of cell disintegration, but is active cell death programmed by the genes of a

cell in order to maintain the life of an individual. Apoptosis plays an important role not only in the formation of a body in the developmental process, but also in normal cell turnover, the maintenance of the nervous system, the establishment of the immune system and the like in a mature individual in order to control the cellular society (see Non Patent Documents 3 and 4). Moreover, it has become clear that apoptosis is involved not only in basic life phenomena, but also closely involved in the onset of various diseases, for example cancer, autoimmune diseases, viral infectious diseases such as AIDS and neurodegenerative diseases such as Alzheimer disease (see Non Patent Document 5).

[0003]

Apoptosis is caused by various apoptosis-inducing factors under physiological and pathological conditions, and is defined by morphological changes and biochemical changes characteristic in apoptotic cells. Apoptosis is distinguished from necrosis, which is a passive disintegration process wherein normal cells that have received extreme injuries, such as from burns or bruises, die.

[0004]

Factors for apoptosis induction include, for example, biological factors such as signals from hormones or cytokines, and removal of growth factors, as well as physical factors such as radioactive rays and heat, and chemical factors such as drugs. The mechanism varies depending on apoptosis-inducing factors. Finally, through a common process mainly comprising DNA fragmentation, cell death occurs.

[0005]

Apoptosis is a form of physiological cell death essential for normal development and differentiation, and occurs in individual cells, for example during the cell turnover in normal biological tissue. Accordingly, excessive suppression of apoptosis causes many functional disorders.

[0006]

Specific examples of such disorders resulting from apoptosis suppression include cancer, proliferative dermatosis, chronic rheumatoid arthritis, HIV infection, hepatitis and renal diseases. There are currently no effective therapeutic agents against these disorders, and agents for treating and improving such conditions, which have a high clinical usefulness, have been desired.

[0007]

In the meantime, it is recently known that microorganisms have a mechanism which is referred to as the quorum sensing system as one of their various recognition and responsive functions. By the mechanism, microorganisms recognize their number in their

environment and respond. In this signalling mechanism, a substance called an autoinducer (AI) is involved. Signalling among microorganisms is conducted via the autoinducer, thereby regulating wide-ranging biochemical and physiological functions such as the promotion of gene transcription activity, the expression of pathogenicity and the production of antibiotics. This quorum sensing has been discovered in many gram-negative bacteria. As a typical autoinducer, an acylated homoserine lactone has been reported. Furthermore, acylated homoserine lactone has been revealed to be involved in a wide variety of activities of microorganisms. As these activities, production of exoenzymes in *Erwinia carotovora*, which is a plant pathogen, and *Pseudomonas aeruginosa*, which is a causative bacterium of cystic fibrosis, and introduction of Ti plasmid from *Agrobacterium tumefaciens* to a plant are known.

[0008]

However, regarding the effect of acylated homoserine lactone on activities and the like of animal cells, very little is known. Further, it has been completely unknown that an acylated homoserine lactone has an effect on apoptosis in cells.

[0009]

[Non Patent Document 1] Br. J. Cancer 26, 239-257, 1972

[Non Patent Document 2] J. Pathol. 111, 85-94, 1973

[Non Patent Document 3] Science 154, 605-612, 1966

[Non Patent Document 4] Rev. Cell. Biol. 7, 663-698, 1991

[Non Patent Document 5] Lancet 341, 1251-1254, 1993

[0010]

[Problems to Be Solved by the Invention]

An object of the present invention is to provide an agent for inducing apoptosis useful for prevention and/or treatment of disorders resulting from apoptosis suppression.

[0011]

[Means to Solve the Problems]

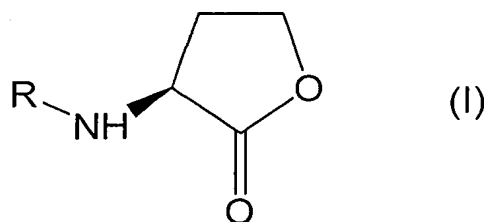
As a result of intensive studies to achieve the above object, we have discovered that acylated homoserine lactone inhibits the activity of Akt, which is an enzyme essential for cell survival, and effectively induces apoptosis, thereby completing the invention.

[0012]

That is, the present invention encompasses the following inventions.

(1) An Akt inhibitor comprising a compound represented by formula I:

[Formula 3]



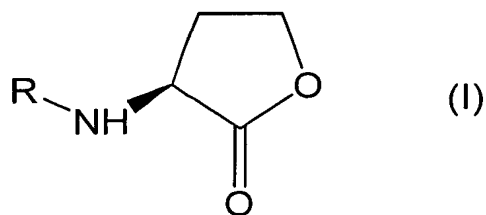
[wherein R is C<sub>4-30</sub> linear or branched acyl, which may be substituted].

(2) The Akt inhibitor of (1), wherein R is C<sub>4-30</sub> linear or branched acyl having oxo at position 3.

(3) An agent for inducing apoptosis, comprising an Akt inhibitor of (1) or (2).

(4) A method of inducing apoptosis in cells, comprising using a compound represented by formula I:

[Formula 4]



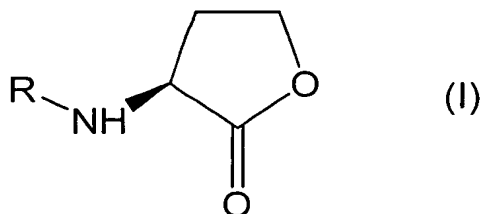
[wherein R is as defined in claim 1].

[0013]

[Mode for Carrying out the Invention]

In the present invention, a compound represented by formula I:

[Formula 5]



is referred to as acylated homoserine lactone.

[0014]

In formula I, R represents C<sub>4-30</sub>, preferably C<sub>8-20</sub> and more preferably C<sub>10-14</sub> linear or branched acyl, which may be substituted. Examples of a substituent include hydroxyl, oxo and methyl. Unsubstituted saturated aliphatic acyl and saturated aliphatic acyl having oxo at position 3 are preferred. In addition, a linear form is preferred.

[0015]

Specific examples of R include, but are not specifically limited to, butyryl, 3-oxobutyryl, 3-hydroxybutyryl, isobutyryl, valeryl, 3-oxovaleryl, isovaleryl, 3-oxoisovaleryl, pivaloyl, hexanoyl, 3-oxohexanoyl, heptanoyl, 3-oxoheptanoyl, octanoyl, 3-oxooctanoyl, nonanoyl, 3-oxononanoyl, decanoyl, 3-oxodecanoyl, undecanoyl, 3-oxoundecanoyl, lauroyl, 3-oxododecanoyl, tridecanoyl, 3-oxotridecanoyl, tetradecanoyl, 3-oxotetradecanoyl, pentadecanoyl, 3-oxopentadecanoyl, palmitoyl, 3-oxopalmitoyl, heptadecanoyl, 3-oxoheptadecanoyl, stearoyl, 3-oxostearoyl, nonadecanoyl, 3-oxononadecanoyl, icosanoyl, 3-oxoicosanoyl, triacontanoyl, 3-oxotriacontanoyl, myristoyl, 3-oxomyristoyl and pyruvoyl.

[0016]

Specific examples of acylated homoserine lactone represented by formula I include, but are not specifically limited to, N-butyryl-L-homoserine lactone, N-(3-oxobutyryl)-L-homoserine lactone, N-(3-hydroxybutyryl)-L-homoserine lactone, N-isobutyryl-L-homoserine lactone, N-valeryl-L-homoserine lactone, N-(3-oxovaleryl)-L-homoserine lactone, N-isovaleryl-L-homoserine lactone, N-(3-oxoisovaleryl)-L-homoserine lactone, N-pivaloyl-L-homoserine lactone, N-hexanoyl-L-homoserine lactone, N-(3-oxohexanoyl)-L-homoserine lactone, N-heptanoyl-L-homoserine lactone, N-(3-oxoheptanoyl)-L-homoserine lactone, N-octanoyl-L-homoserine lactone, N-(3-oxooctanoyl)-L-homoserine lactone, N-nonanoyl-L-homoserine lactone, N-(3-oxononanoyl)-L-homoserine lactone, N-decanoyl-L-homoserine lactone, N-(3-oxodecanoyl)-L-homoserine lactone, N-undecanoyl-L-homoserine lactone, N-(3-oxoundecanoyl)-L-homoserine lactone, N-lauroyl-L-homoserine lactone, N-(3-oxododecanoyl)-L-homoserine lactone, N-tridecanoyl-L-homoserine lactone, N-(3-oxotridecanoyl)-L-homoserine lactone, N-tetradecanoyl-L-homoserine lactone, N-(3-oxotetradecanoyl)-L-homoserine lactone, N-pentadecanoyl-L-homoserine lactone, N-(3-oxopentadecanoyl)-L-homoserine lactone, N-palmitoyl-L-homoserine lactone, N-(3-oxopalmitoyl)-L-homoserine lactone, N-heptadecanoyl-L-homoserine lactone, N-(3-oxoheptadecanoyl)-L-homoserine lactone, N-stearoyl-L-homoserine lactone, N-(3-oxostearoyl)-L-homoserine lactone, N-nonadecanoyl-L-homoserine lactone, N-(3-oxononadecanoyl)-L-homoserine lactone, N-icosanoyl-L-homoserine lactone, N-(3-oxoicosanoyl)-L-homoserine lactone, N-triacontanoyl-L-homoserine lactone, N-(3-oxotriacontanoyl)-L-homoserine lactone, N-myristoyl-L-homoserine lactone, N-(3-oxomyristoyl)-L-homoserine lactone and N-pyruvoyl-L-homoserine lactone.

[0017]

In particular, N-(3-oxodecanoyl)-L-homoserine lactone, N-(3-oxoundecanoyl)-L-homoserine lactone, N-(3-oxododecanoyl)-L-homoserine lactone, N-(3-oxotridecanoyl)-L-homoserine lactone and N-(3-oxotetradecanoyl)-L-homoserine lactone are preferred.

[0018]

The acylated homoserine lactone of the present invention can be synthesized by, for example, forming an amide bond between aliphatic carboxylic acid or the ester thereof and cyclic amino acid. Furthermore, acylated homoserine lactone can be synthesized by a method described in, for example, Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, G. S. A. B. Stewart, P. Williams, and B. W. Bycroft, *J. Antibiot.*, 46, 441-454, 1993, Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate, *Nature*, 362, 446-448, 1993, Schaefer A.L., B. L. Hanzelka, A. Eberhard, and E. P. Greenberg, *J. Bacteriol.*, 178, 2897-2901, 1996, and Gao, J. G. and E. A. Meighen. *J. Bacteriol.*, 175, 3856-3862, 1993.

[0019]

Furthermore, the acylated homoserine lactone of the present invention is biosynthesized by microorganisms, so that it can be separated and purified using a method conventionally employed in the art from the culture product obtained by culturing microorganisms.

[0020]

Acylated homoserine lactone was reacted with various animal cells, and then Akt activity in the cells was determined by the Western blotting method. As a result, we have discovered that specific acylated homoserine lactone effectively inhibits the phosphorylation of Akt.

[0021]

In the present invention, Akt is serine/threonine kinase, which is activated in the downstream of PI3K (phosphatidylinositide 3-OH kinase), and PI3K-Akt pathway is known as one of the survival signalling pathways. In addition, Akt has also been reported to have activity to inhibit apoptosis in cells (JP Patent Publication (Kohyo) No. 2002-528390 A).

[0022]

In the present invention, "apoptosis" has a meaning generally used in the art, and specifically refers to cell death, which is actively induced by a cell itself under physiological conditions. The morphology of apoptosis is characterized by chromosome aggregation in cell nuclei, fragmentation of cell nuclei, disappearance of microvilli of cell cortex, and chromatin condensation. Cells shrink, and then are immediately incorporated by surrounding cells such as macrophages without extracellular releasing of cell contents. Thus, apoptosis causes no inflammation and has no effect on surrounding cells. On the



other hand, necrosis caused by environmental deterioration greatly differs from apoptosis in that cell contents are released.

[0023]

Because of the above characteristics, apoptosis in cells can be detected by various methods. Examples of a method for detecting apoptosis include, but are not specifically limited to, a method that involves staining using, for example, DNA binding fluorescent dye such as aminobenzimide (e.g., Hoechst 33342, 33582 and 333258), and then observing chromatin condensation under a fluorescence microscope; a method that involves extracting and separating fragmented DNA by centrifugation or the like, and then quantifying by colorimetry or the like; a method that involves detecting fragmented DNA as "ladder" in agarose gel electrophoresis; and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) method that involves histochemically detecting fragmented DNA.

[0024]

Examples of cells into which apoptosis can be induced using the agent for inducing apoptosis of the present invention include, but are not specifically limited to, plant cells and animal cells. Apoptosis can be particularly effectively induced in animal cells.

[0025]

According to the present invention, it was shown that acylated homoserine lactone effectively inhibits Akt activity in cancer cells. Thus, acylated homoserine lactone can be used as an Akt inhibitor for cancer cells. Furthermore, when acylated homoserine lactone was applied to cancer cells, reduction in viability of the cancer cell, chromatin condensation and the like were observed. Thus it was also shown that the acylated homoserine lactone of the present invention effectively induces apoptosis in cancer cells. Hence, the acylated homoserine lactone of the present invention can be used as an active ingredient of an anticancer agent.

[0026]

In the present invention, "Akt inhibitor" refers to an agent that inhibits the activation of Akt, that is, the phosphorylation of Akt, and an agent that inactivates activated Akt. Examples of such an agent include an agent inhibiting the activation of Akt in animal cells, an agent inhibiting normal localization of Akt within cells, an agent inhibiting the action of a substance that activates Akt and an agent degrading an Akt-activating substance.

[0027]

Furthermore, acylated homoserine lactone represented by formula I differs in its Akt-inhibiting activity depending on types of R groups, so that the apoptosis-inducing

effect can be regulated by replacing or modifying the R group. In addition, it is considered that by the use of a combination of a plural number of types of acylated homoserine lactone having different R groups, changes in Akt activity can be controlled and the pharmacological effects thereof can be regulated.

[0028]

Examples of a disease that can be treated using an agent for inducing apoptosis comprising as an active ingredient the acylated homoserine lactone of the present invention include, but are not specifically limited to, diseases caused by the suppression of apoptosis, such as cancer, which is present in tissue selected from the group consisting of the ovary, breast, pancreas, skin, lung, brain, kidney, liver, epipharynx, central nervous system, prostate, large bowel, colon, rectum, uterine cervix and endometrium, and proliferative dermatosis, rheumatoid arthritis, HIV infection, hepatitis and renal disorders. In particular, the agent for inducing apoptosis is appropriately used in treating cancer of the digestive system, such as colon cancer, and cutaneous cancer.

[0029]

In the agent for inducing apoptosis of the present invention, acylated homoserine lactone may be a hydrate. Furthermore, acylated homoserine lactone according to the present invention may be in a free form, or a pharmacologically acceptable salt thereof. When a salt is used, specific examples of a salt include an addition salt of alkaline metal, an addition salt of alkaline earth metal, an ammonium salt and an addition salt of amine. However, acylated homoserine lactone in a free form is more preferred.

[0030]

Next, examples of a dosage form of the acylated homoserine lactone of the present invention include oral preparations such as powder, fine grain agents, granules, tablets, coated tablets and capsules, external preparations such as ointments and patches, suppositories and preparations for injection. Upon formulation, the preparation can be produced using a conventional carrier for formulation by a standard method.

[0031]

Specifically, when an oral preparation is produced, the acylated homoserine lactone and an excipient, and if necessary an antioxidant, a binder, a disintegrating agent, a lubricant, a coloring agent, a flavoring agent or the like is added, and then the mixture is formulated into powder, a fine grain agent, a granule, a tablet, a coated tablet, a capsule or the like by a standard method.

[0032]

Examples of an excipient that is used herein include lactose, corn starch, sucrose, glucose,

mannitol, sorbit, crystalline cellulose and silicon dioxide. Examples of a binder that is used herein include polyvinyl alcohol, polyvinyl ether, methyl cellulose, ethyl cellulose, gum arabic, gum tragacanth, gelatine, shellac, hydroxypropylmethylcellulose, hydroxypropylcellulose, polyvinylpyrrolidone, polypropyleneglycol · polyoxyethylene · block polymer and meglumine. Examples of a disintegrating agent that is used herein include starch, agar, gelatine powder, crystalline cellulose, calcium carbonate, sodium bicarbonate, calcium citrate, dextrin, pectin and carboxymethyl cellulose · calcium. Examples of a lubricant that is used herein include talc, polyethylene glycol, silica and hardened vegetable oil. Examples of a coloring agent that is used herein include coloring agents that are capable of being added to pharmaceuticals. Examples of a flavoring agent that is used herein include cocoa powder, menthol, aroma powder, mentha oil, borneol and cinnamon powder. These tablets and granules may be sugar-coated, or may be appropriately coated with other substances if necessary.

[0033]

When a preparation for injection is produced, a pH modifier, a solvent and an isotonicizing agent, as well as, if necessary, a solubilizer, a stabilizer, an antioxidant or the like are added to the acylated homoserine lactone, thereby formulating it by a standard method.

[0034]

A method for producing an external preparation is not limited, and it can be produced by a standard method. Specifically, as a material for a base to be used upon formulation, various materials that are generally used for pharmaceuticals, quasi-drugs, cosmetics and the like can be used.

[0035]

Specific examples of a material for a base to be used herein include animal and vegetable oils, mineral oil, ester oil, waxes, higher alcohols, fatty acids, silicone oil, surfactants, alcohols, polyalcohols, water-soluble polymers, clay minerals and purified water. Furthermore, if necessary, a pH modifier, an antioxidant, a chelating agent, an antiseptic and antifungal agent, a coloring agent, an odorant or the like can be added. However, the materials for the base used in the external preparation of the present invention are not limited thereto. Furthermore, if necessary, a constituent such as a blood stream accelerator, an antimicrobial agent, an anti-inflammatory agent, a cell activator, vitamins, amino acids, a moisturizing agent, a keratolytic agent or the like can be incorporated. In addition, the above material for the base is added in an amount suitable to achieve a concentration that is generally defined for the production of an external preparation.

[0036]

The clinical dose of acylated homoserine lactone, which is an active ingredient of the agent for inducing apoptosis of the present invention, differs depending on animals, to which the inducer is administered, symptom, seriousness, age, body weight, complication and the like. It also differs depending on salt types and routes of administration. Generally, the clinical dose for an adult per day is between 1  $\mu$ g and 1 g, preferably between 10  $\mu$ g and 200 mg, and more preferably between 20  $\mu$ g and 20 mg.

[0037]

A target, to which the agent for inducing apoptosis of the present invention is to be administered, is not specifically limited, as long as it is an animal. Examples of such animals include mammals such as horses, dogs, mice, guinea pigs and rats. In particular, the inducer is preferably used for a human.

[0038]

[Example]

In the following examples, experimental materials described below were used, unless otherwise specified.

[0039]

Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), non essential amino acid (NEAA) and penicillin/streptomycin (P/S) were purchased from SIGMA and used for cell culture. Dimethyl sulfoxide (DMSO) of Wako Pure Chemical Industries, Ltd was used as a solvent for N-acyl-L-homoserine lactone (AHL). PD98059 and SB203580, the kinase inhibitors, were purchased from CALBIOCHEM. For protein quantification, BCA Protein Assay Reagent (PIERCE) was used. Antibodies used herein were those of the following companies. An anti-phospho-Akt (Ser473) antibody, an anti-cleaved caspase-3 antibody, an anti-cleaved caspase-9 (D330) antibody, an anti-cleaved PARP (D214) antibody, and an HRP-labeled anti-rabbit IgG antibody were from Cell Signaling. An HRP-labeled anti-mouse IgG antibody was from SANTA CRUZ BIOTECHNOLOGY. An anti- $\alpha$ -tubulin antibody (Ab-1) was from CALBIOCHEM.

[0040]

#### Example 1 Analysis of Akt activity by Western blot

(1) Changes in Akt activity in CaCo-2 cells by stimulation with acylated homoserine lactone

CaCo-2, the carcinoma cell line of human colon cancer, was purchased from American Type Culture Collection (ATCC), and cultured at 37°C in the presence of 5% CO<sub>2</sub> using a DMEM medium containing 10% FCS, 1% NEAA, and 1% P/S (100 units/ml penicillin and

100 µg/ml streptomycin).

[0041]

$6 \times 10^5$  CaCo-2 cells were inoculated per dish using 6 cm dishes (NUNC), washed twice with phosphate buffered saline (PBS) (-), and then cultured for 24 hours in a serum-free DMEM medium containing 1% NEAA. Subsequently, at a final concentration of 100 µM, homoserine lactone hydrochloride, N-butyryl-L-homoserine lactone, N-(3-oxohexanoyl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone were each added, and then each solution was allowed to react for 10 minutes. In addition, purified acylated homoserine lactone that was used herein had been dissolved in DMSO in a concentration 400 times higher than the target final concentration, and then subjected to filter sterilization ( $\phi$  0.22 µm). DMSO at a final concentration of 0.25% was used as a control. In addition,  $\alpha$ -tubulin was used as an internal standard.

[0042]

After reaction, the product was washed twice with cold PBS (-), and then frozen at -80°C. The cells were thawed on ice, 200 µl of a lysis buffer (50 mM HEPES, pH7.5, 50 mM NaCl, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 1% Triton X-100, 10% glycerine, 10 mM sodium pyrophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) was added, and then the cells were scraped off using a scraper. The cells were allowed to stand on ice for 20 minutes, and then centrifuged at 14,000 rpm at 4°C for 15 minutes, thereby collecting the supernatant. Concentration of the collected protein was measured, and protein equivalent to 50 µg was subjected to SDS-PAGE. After electrophoresis, the product was blotted on a PVDF membrane. When phospho-Akt was measured, blocking was carried out with 5% skim milk/Tris-buffered saline containing 0.1% Tween 20 (TBS-T), and the anti-phospho-Akt (Ser437) antibody and the HRP-labeled anti-rabbit IgG antibody were used as a primary antibody and a secondary antibody, respectively. For  $\alpha$ -tubulin used as an internal standard, blocking was carried out with 5% bovine serum albumin (BSA)/TBS-T, and the anti- $\alpha$ -tubulin (Ab-1) and the HRP-labeled anti-mouse IgG antibody were used as a primary antibody and a secondary antibody, respectively. For band detection, Renaissance™ Western Blot Chemiluminescence 255861 Reagent Plus (NEN) or Western Blotting Luminol Reagent (SANTA CRUZ BIOTECHNOLOGY) was used. Figure 1 shows the results. In addition in the figure that is described below, HSL means homoserine lactone hydrochloride, C4-HSL means N-butyryl-L-homoserine lactone, 3-oxo-C6-HSL means N-(3-oxohexanoyl)-L-homoserine lactone, and 3-oxo-C12-HSL means N-(3-oxododecanoyl)-L-homoserine lactone.

[0043]

In CaCo-2 cells, Akt is normally in a phosphorylated (activated) form. However, the level of phosphorylated Akt significantly decreased only when N-(3-oxododecanoyl)-L-homoserine lactone was added. Specifically, it was shown that N-(3-oxododecanoyl)-L-homoserine lactone inhibits the activity of Akt.

[0044]

(2) N-(3-oxododecanoyl)-L-homoserine lactone concentration-dependent changes in Akt activity in CaCo-2 cells

After cell culture, N-(3-oxododecanoyl)-L-homoserine lactones with final concentrations of 0  $\mu$ M (0.25% DMSO), 1  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M were each added. Akt activity was analyzed by Western blot in a manner similar to that used in (1), except for carrying out reaction for 10 minutes (Figure 2).

[0045]

Figure 3 shows the results of quantifying the band intensities in Figure 2 by LAS-1000 and converting the results into numerical data. At concentrations of 10  $\mu$ M or more, the levels of phosphorylated Akt significantly decreased. At 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M, Akt activity decreased to 79%, 31% and 14%, respectively, of that in a case where no stimulation was provided.

[0046]

From the above results, it was shown that in CaCo-2 cells, Akt activity is significantly inhibited by 10  $\mu$ M or more acylated homoserine lactones.

[0047]

(3) Changes with time in Akt activity in CaCo-2 cells stimulated with N-(3-oxododecanoyl)-L-homoserine lactone

After cell culture, N-(3-oxododecanoyl)-L-homoserine lactones with final concentrations of 30  $\mu$ M and 100  $\mu$ M were each added. For each case, Akt activity was measured by Western blot in a manner similar to that in (1), except for carrying out stimulation with acylated homoserine lactone in a short time course of 0, 5, 10, 30 and 60 minutes (Figure 4), and in a long time course of 0, 10 minutes, 60 minutes, 2 hours, 4 hours, 6 hours, 8 hours and 10 hours (Figure 5). In the case of stimulation at 30  $\mu$ M, the inhibition of Akt activity peaked at 5 minutes, and lasted until 60 minutes after stimulation, and then returned to the base level within 2 hours. On the other hand, in the case of stimulation at 10  $\mu$ M, the inhibition of Akt activity peaked at 5 minutes and lasted until 6 hours after stimulation.

[0048]

(4) Changes in Akt activity in porcine vascular endothelial cells stimulated with N-(3-oxododecanoyl)-L-homoserine lactone

Porcine vascular endothelial cells (PECs) were cultured at 37°C in the presence of 5% CO<sub>2</sub> using a DMEM medium containing 10% FCS, 1% NEAA and 1% P/S (100 units/ml penicillin and 100 µg/ml streptomycin).

[0049]

6×10<sup>5</sup> PECs were inoculated per dish using 6 cm dishes (NUNC), washed twice with phosphate buffered saline (PBS) (-), and then cultured for 24 hours in a serum-free DMEM medium containing 1% NEAA. Subsequently, at a final concentration of 100 µM, homoserine lactone hydrochloride, N-butyryl-L-homoserine lactone, N-(3-oxohexanoyl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone were each added, and then each solution was allowed to react for 10 minutes. Similarly to (1), the effect of acylated homoserine lactone on Akt activity in PECs were examined by Western blotting. Figure 6 shows the results.

[0050]

The result of Western blotting revealed that N-(3-oxododecanoyl)-L-homoserine lactone significantly decreases Akt activity in PECs.

[0051]

The above results suggest that specific acylated homoserine lactones induce apoptosis in PECs.

[0052]

#### Example 2 Evaluation of viability by Trypan blue staining

2×10<sup>5</sup> CaCo-2 cells were inoculated in a 3.5 cm dish (NUNC), washed twice with PBS (-), and then cultured in a serum-free DMEM medium containing 1% NEAA for 24 hours. Various agents [N-(3-oxododecanoyl)-L-homoserine lactones with final concentrations between 1 and 100 µM] were added, followed by 12 hours of culture. 0.25% DMSO was used as a control. Subsequently, the supernatant and the cells removed from the dish using trypsin-EDTA (TE) were centrifuged at 800 rpm at room temperature for 5 minutes. The supernatant was removed, and then dilution was carried out with 200 µl of DMEM. 50 µl of the dilution was taken in an Eppen tube, an equivalent volume of Trypan blue stain was added, and then the cell count was measured using a hemacytometer. At this time, the sum of the dead cell count and the viable cell count to be measured was determined to be 200 cells or more.

[0053]

A significant decrease was observed in viability in a manner depending on the concentration of N-(3-oxododecanoyl)-L-homoserine lactone. With 10 µM, 30 µM and 100 µM N-(3-oxododecanoyl)-L-homoserine lactones, the viabilities respectively decreased

to 90%, 55% and 51% (Figure 13). These results showing that the viability was significantly decreased at concentrations of 30  $\mu$ M or more, are consistent with the results showing that increased the inhibition of Akt activity were significantly observed in Western blot analysis at around 30  $\mu$ M. Moreover, the viabilities in the case of adding 0.25% DMSO were both 94%. Thus, it is considered that since stimulation with 1  $\mu$ M N-(3-oxododecanoyl)-L-homoserine lactone resulted in a viability of 94 %, the effect of DMSO on viability can be ignored.

[0054]

Example 3 Observation of chromatin condensation and determination of apoptosis by Hoechst33342 staining

To determine whether the cell death of CaCo-2 observed in Example 2 were due to apoptosis or necrosis, morphological evaluation was carried out using Hoechst33342 fluorescent dye for chromatin staining.

[0055]

$2 \times 10^4$  CaCo-2 cells were inoculated on an 8-well culture slide (Collagen I Cellware BioCoat, Becton Dickinson), washed twice with PBS (-), and then cultured in a serum-free DMEM medium containing 1% NEAA for 24 hours. 3-oxododecanoyl homoserine lactone was added at each concentration (1 to 100  $\mu$ M). After 12 hours of culture, the cells were fixed by 4% paraformaldehyde-3% sucrose/PBS, chromatin staining was carried out using Hoechst 33342 fluorescent dye, and then the cells were observed under a fluorescence microscope. With a magnification of x400, 5 visual fields were selected at random. Apoptotic cells and normal cells were counted and the proportion of apoptotic cells was calculated. These steps were denoted as n=1, and conducted 3 times or more.

[0056]

The apoptotic cells exhibiting chromatin condensation were observed at concentrations of 10  $\mu$ M or more of N-(3-oxododecanoyl)-L-homoserine lactone. However, in the cases of 0.25% DMSO and 1  $\mu$ M N-(3-oxododecanoyl)-L-homoserine lactone, only normal cells were observed (Figure 8). Figure 9 shows the result of quantifying apoptosis by counting apoptotic cells in which chromatin condensation was observed. The proportions of apoptotic cells to the total cell count were 15%, 39% and 50% respectively in the cases of 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M N-(3-oxododecanoyl)-L-homoserine lactones. These values are also consistent with the decreases in viabilities. Therefore, it was determined that cell death of CaCo-2 resulting from N-(3-oxododecanoyl)-L-homoserine lactone was due to apoptosis.

[0057]



Example 4 Evaluation of apoptosis by DNA fragmentation

In another method for determining whether the cell death of CaCo-2 observed in Example 2 was due to apoptosis or necrosis, DNA fragmentation was evaluated.

[0058]

$5 \times 10^6$  CaCo-2 cells were inoculated per 15 cm dish (NUNC), washed twice with PBS (-), and then cultured in a serum-free DMEM medium containing 1% NEAA for 24 hours. N-(3-oxododecanoyl)-L-homoserine lactone was added at each concentration (1  $\mu$ M to 100  $\mu$ M), followed by 12 hours of culture. Subsequently, the supernatant and the cells removed from the dish using TE were centrifuged at room temperature at 800 rpm for 5 minutes, and the supernatant was removed. The cells were suspended with 300  $\mu$ l of PBS (-), and then the suspension was centrifuged again at 4°C at 2500 rpm for 5 minutes, and the supernatant was removed. The cells were lysed by the addition of 300  $\mu$ l of a cytolysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100], and then allowed to stand on ice for 10 minutes, thereby extracting DNA fragments. The extract was centrifuged at 4°C at 14000 rpm for 10 minutes. RNase A was added to the obtained supernatant, and then incubation was carried out at 37°C for 1 hour. Proteinase K was further added, and then incubation was carried out at 50°C for 30 minutes. The extract of the DNA fragments was concentrated by ethanol precipitation, and then electrophoresis was carried out using 2% agarose gel. After the gel was stained with ethidium bromide, DNA fragmentation was detected under a UV transilluminator.

[0059]

When the detection of a DNA ladder was conducted for CaCo-2 cells cultured for 12 hours in the presence of each of 0  $\mu$ M, 1  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M N-(3-oxododecanoyl)-L-homoserine lactones, the DNA ladder was observed at the concentrations of 30  $\mu$ M or more (Figure 10).

[0060]

Example 5 Measurement of caspase activity

The activities of caspase (caspase-3 and caspase-9) and PARP (poly-ADP ribose polymerase) were measured in a manner similar to those in Example 1 by Western blot analysis using each of anti-activated (cleaved) caspase antibodies, that is, an anti-cleaved caspase-3 antibody, an anti-cleaved caspase-9 antibody (D330) and an anti-cleaved PARP antibody (D214).

[0061]

N-(3-oxododecanoyl)-L-homoserine lactones with final concentrations of 30  $\mu$ M and 100  $\mu$ M were each added to cultured CaCo-2 cells. Caspase-3, caspase-9 and PARP activities

were analyzed over time by Western blot at 0 hours, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 10 hours after culture. Figure 11 shows the result. Whereas at 30  $\mu$ M, activated caspase-3 began to be detected 1 hour after stimulation and the activity lasted until 4 hours after stimulation, at 100  $\mu$ M the activity peaked at 6 hours and lasted until 10 hours after stimulation. Cleaved caspase-9 and cleaved PARP were detected by reprobing each membrane. Whereas caspase-9 activity was observed to be strong at 1, 2 and 4 hours and its weak activity lasted until 8 hours after stimulation at 30  $\mu$ M, its strong activity lasted until 8 hours after stimulation at 100  $\mu$ M. Regarding cleaved PARP, it also showed behavior similar to that of cleaved caspase-9.

[0062]

Next, 3 hours of reaction with N-(3-oxododecanoyl)-L-homoserine lactone was carried out at concentrations of 0, 1, 10, 30, and 100  $\mu$ M, and then caspase and PARP activities were measured. As shown in Figure 12, only at concentrations of 30  $\mu$ M and 100  $\mu$ M, cleaved caspase and cleaved PARP were detected. These results were also consistent with the result obtained by Western blot analysis showing that the inhibition of Akt activity, decreased viabilities and the appearance of apoptotic cells significantly occurred at 30  $\mu$ M or more.

[0063]

#### [Effect of the Invention]

According to the present invention, apoptosis can be effectively induced in cells, and a useful means is provided for prevention and/or treatment against disorders resulting from apoptosis suppression.

#### [Brief Description of The Drawings]

##### [Figure 1]

Figure 1 shows changes in Akt activity in CaCo-2 cells stimulated with DMSO, homoserine lactone hydrochloride, N-butyryl-L-homoserine lactone, N-(3-oxohexanoyl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone.

##### [Figure 2]

Figure 2 shows N-(3-oxododecanoyl)-L-homoserine lactone concentration-dependent changes in Akt activity in CaCo-2 cells.

##### [Figure 3]

Figure 3 shows the results of quantifying the band intensities of phospho-Akt in Figure 2 by LAS-1000 and converting the results into numerical data.

##### [Figure 4]

Figure 4 shows changes with time in Akt activity in CaCo-2 cells stimulated with

N-(3-oxododecanoyl)-L-homoserine lactone.

[Figure 5]

Figure 5 shows changes with time in Akt activity in CaCo-2 cells stimulated with N-(3-oxododecanoyl)-L-homoserine lactone.

[Figure 6]

Figure 6 shows changes in Akt activity in porcine vascular endothelial cells stimulated with DMSO, homoserine lactone hydrochloride, N-butyryl-L-homoserine lactone, N-(3-oxohexanoyl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone.

[Figure 7]

Figure 7 shows the results of evaluating cell viability in the presence of acylated homoserine lactone by Trypan blue staining.

[Figure 8]

Figure 8 shows the results of determining apoptosis in cells in the presence of acylated homoserine lactone by chromatin condensation using Hoechst 33342 staining.

[Figure 9]

Figure 9 shows the results of quantifying apoptosis by counting apoptotic cells for which chromatin condensation was observed.

[Figure 10]

Figure 10 shows the results of evaluating DNA fragmentation in order to determine whether the death of CaCo-2 cells was due to apoptosis or necrosis.

[Figure 11]

Figure 11 shows the results of measuring the culture-time dependency of caspase activity in the presence of acylated homoserine lactone by Western blot.

[Figure 12]

Figure 12 shows the results of measuring the acylated homoserine lactone concentration dependency of caspase activity by Western blot.

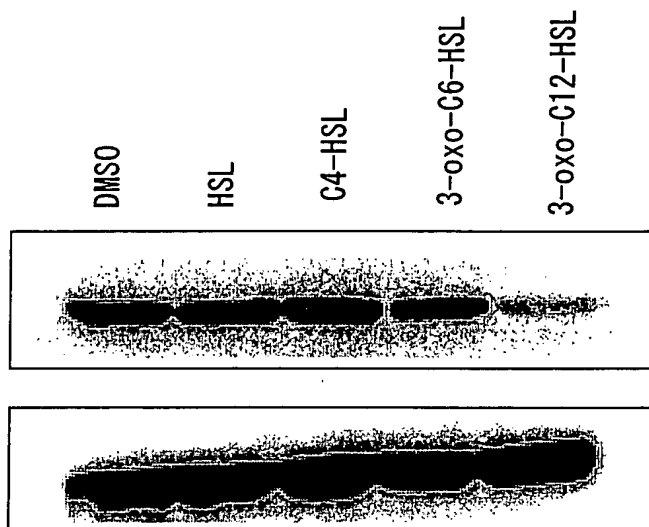
[Name of Document] FIGURES

[Figure 1]

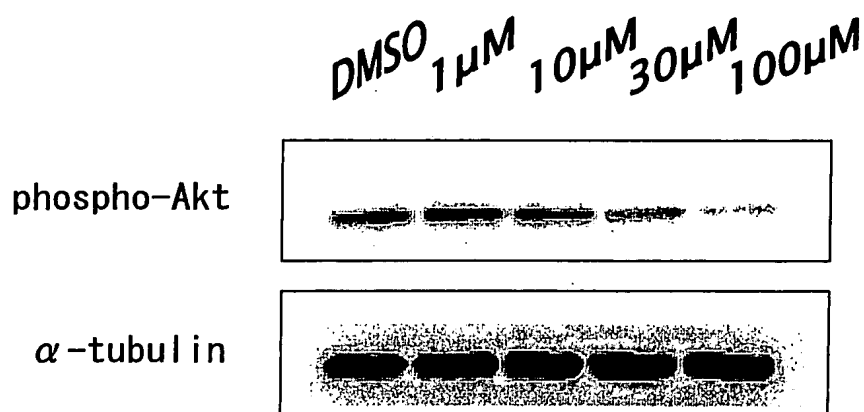


phospho-Akt

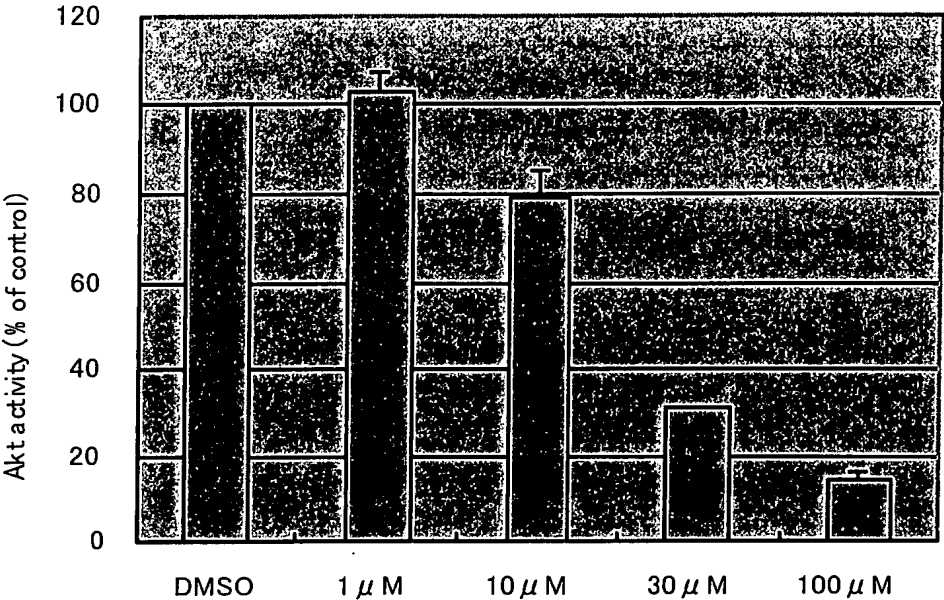
$\alpha$ -tubulin



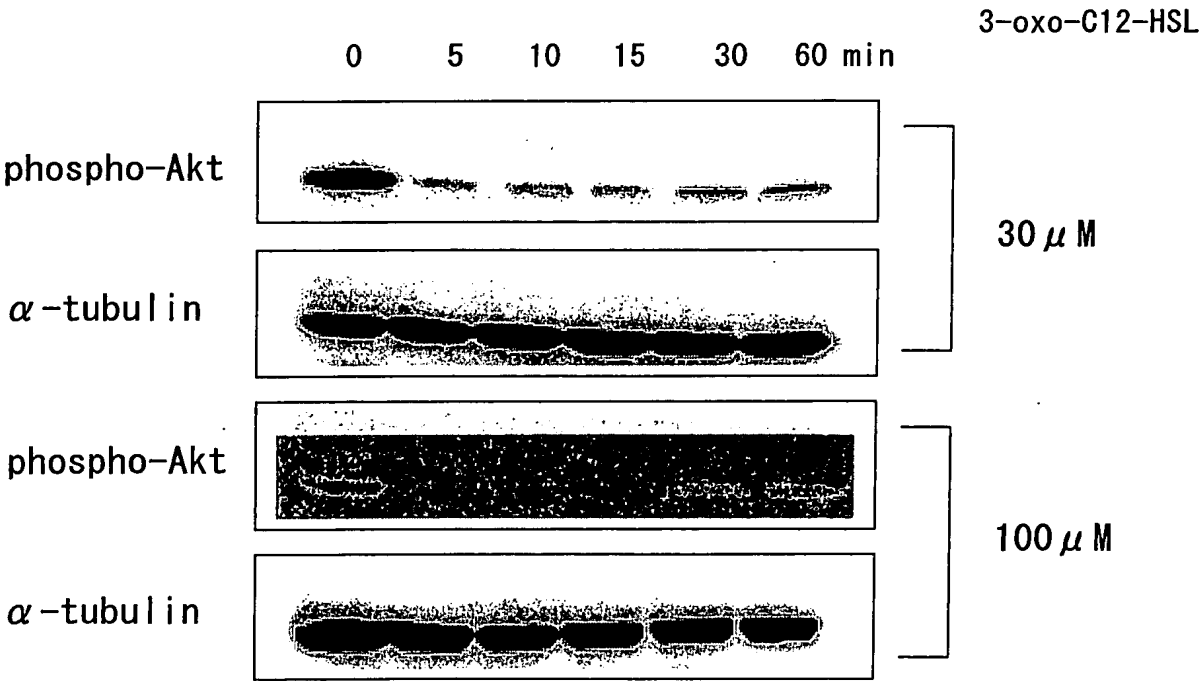
[Figure 2]



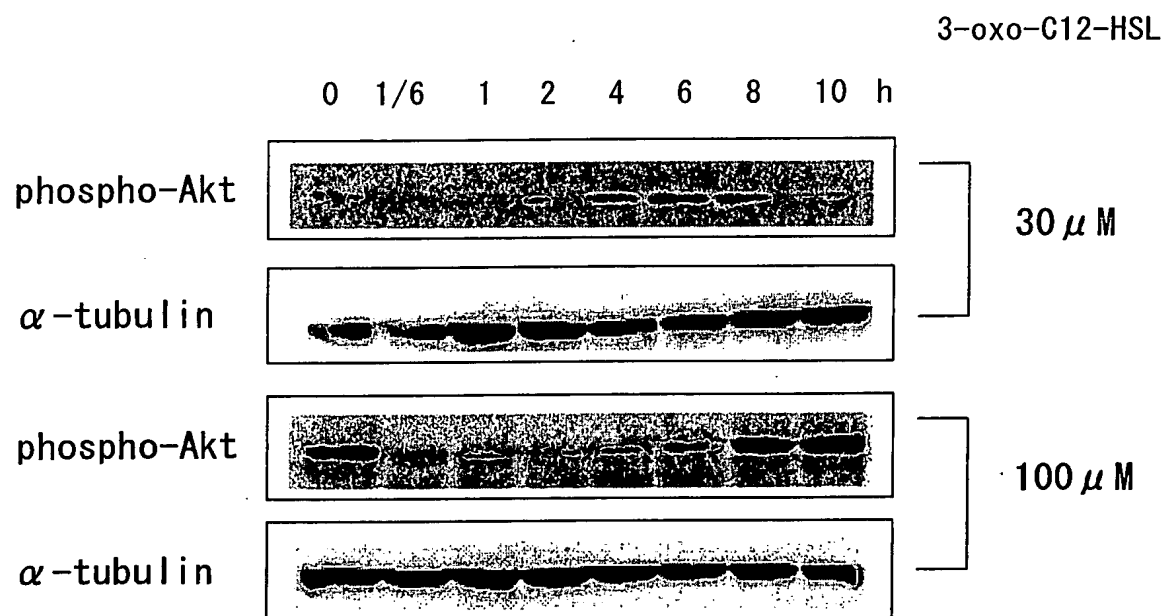
[Figure 3]



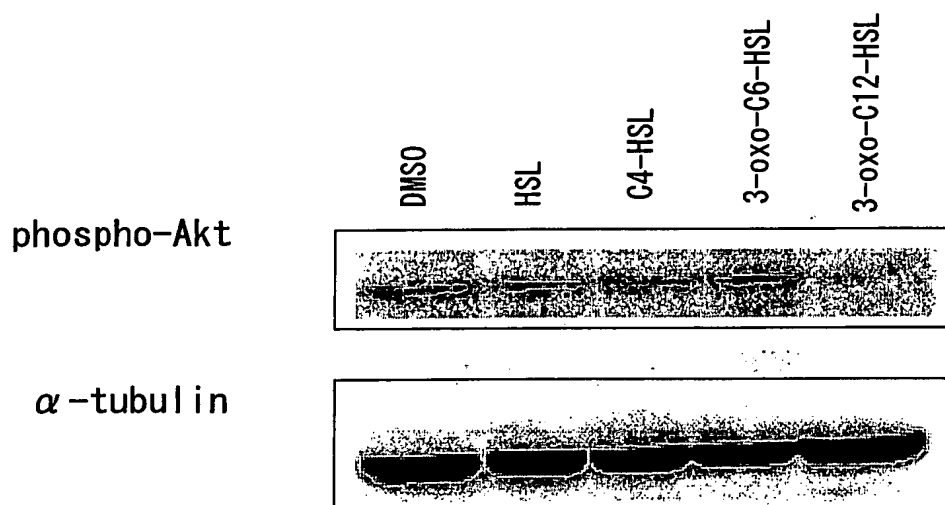
[Figure 4]



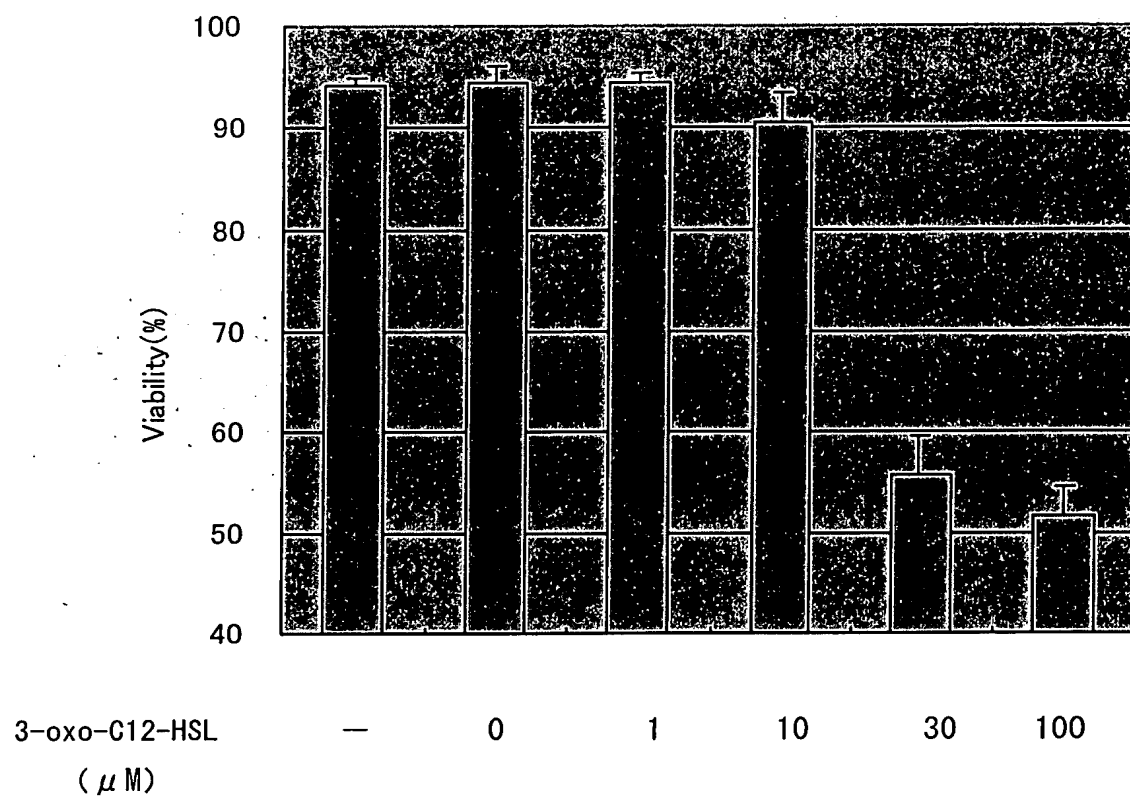
[Figure 5]



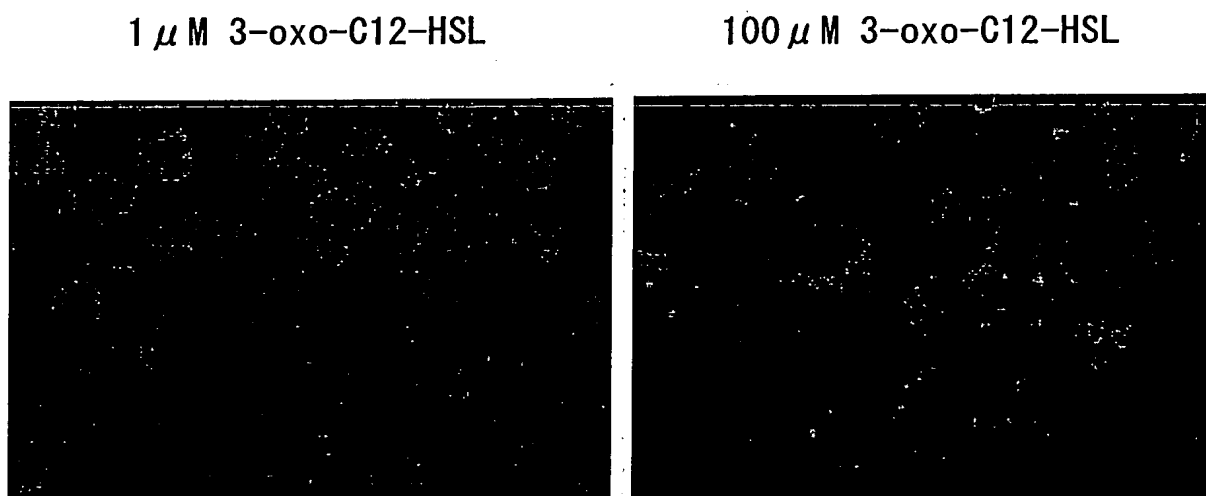
[Figure 6]



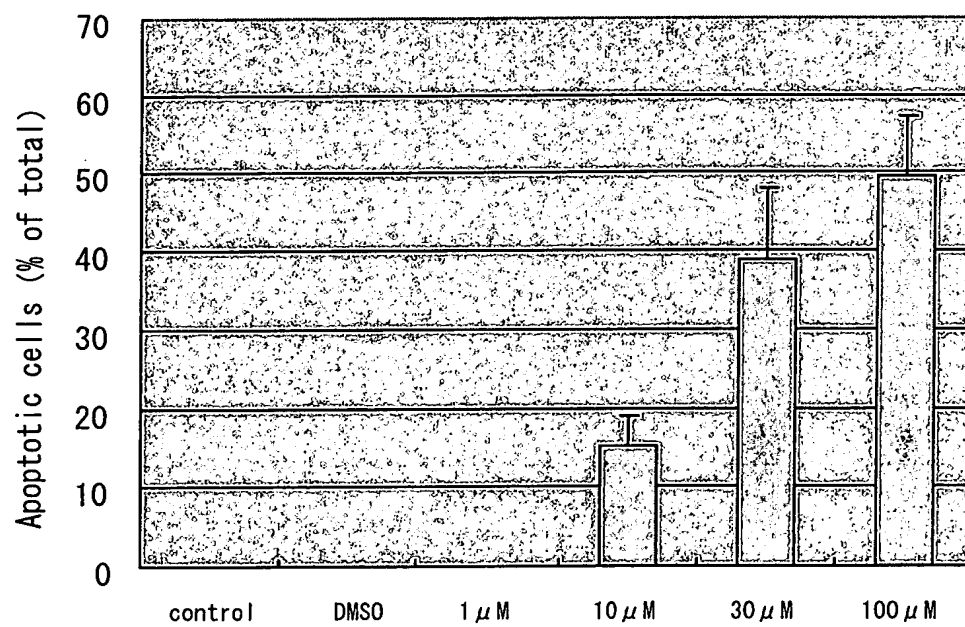
[Figure 7]



[Figure 8]

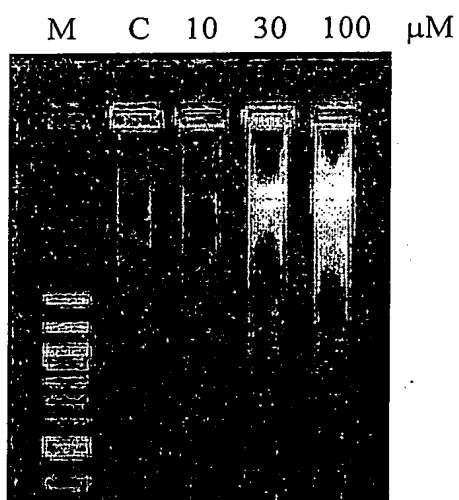


[Figure 9]



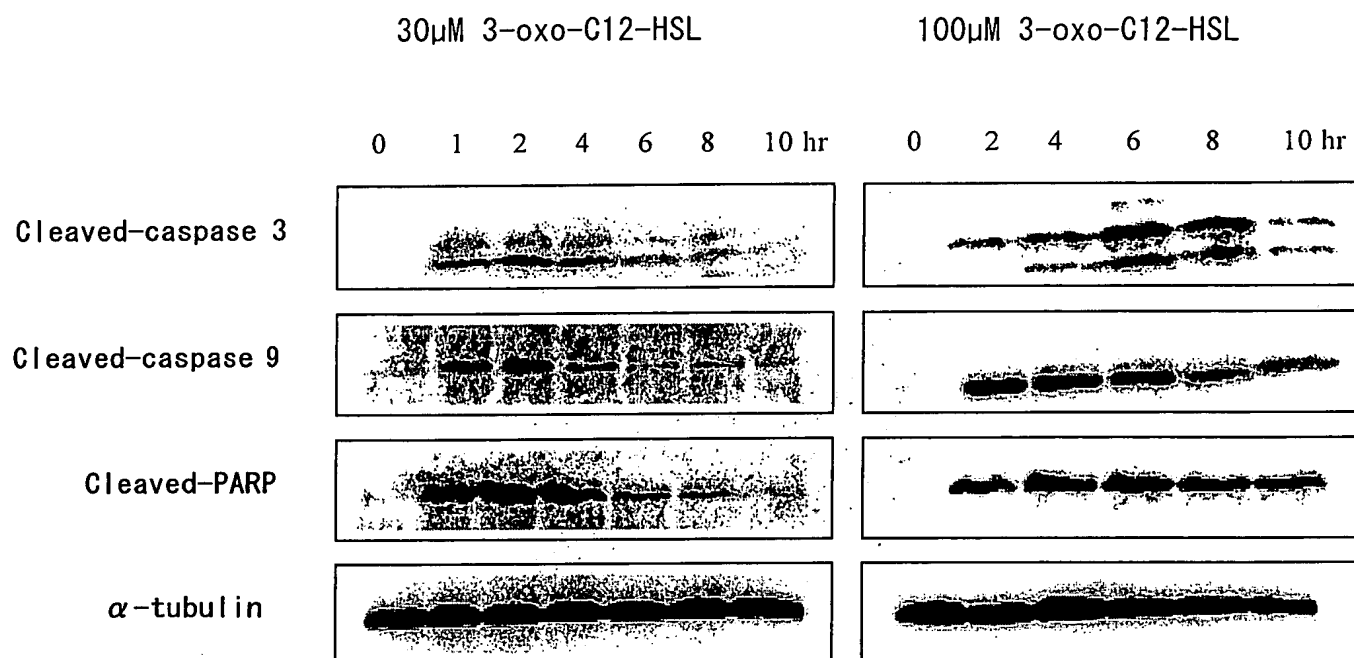


[Figure 10]

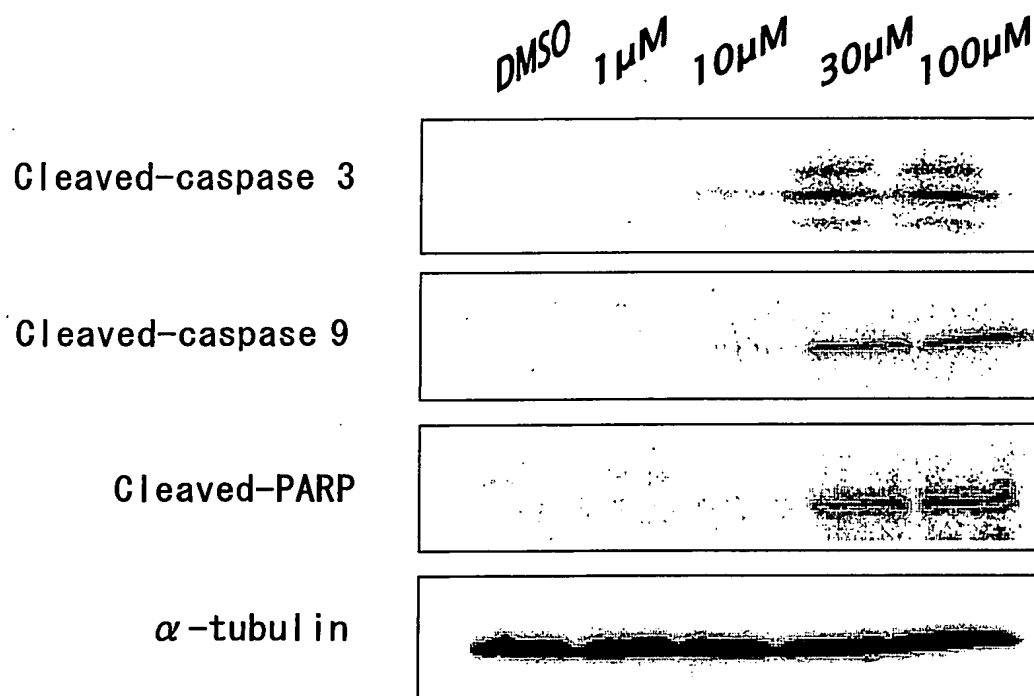


M: marker  
C: control

[Figure 11]



[Figure 12]



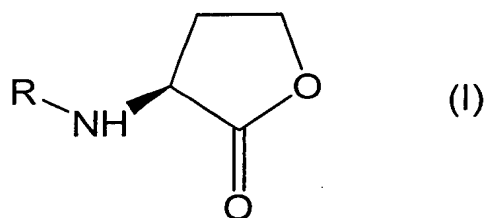
[Name of Document] ABSTRACT

[Abstract]

[Problems] An object of the present invention is to provide an agent for inducing apoptosis useful for prevention and/or treatment of disorders resulting from apoptosis suppression.

[Means for Solution] An Akt inhibitor comprising a compound represented by formula I:

[Formula 1]



[wherein R is C<sub>4-30</sub> linear or branched acyl, which may be substituted].

[Selected Figure] None